ECOTOXICOLOGY AND ENVIRONMENTAL SAFETY 25, 41–47 (1993)

Liver Abnormalities Associated with Chronic Mercury Accumulation in Stranded Atlantic Bottlenose Dolphins

ARNOLD J. RAWSON,* GEOFFREY W. PATTON,* SUZANNE HOFMANN,* GIUSEPPE G. PIETRA,† AND LEW JOHNS†

*Mote Marine Laboratory, Sarasota, Florida 34236; and †Department of Pathology, Hospital of the University of Pennsylvania, Philadelphia, Pennsylvania 19104

Received February 6, 1992

Eighteen stranded Atlantic bottlenose dolphins (*Tursiops truncatus*) examined postmortem were sampled for histologic study. All cases were examined for ferric ion and lipofuscin. Ages were determined from tooth growth layers. Electron microscopic (EM) examination and X-ray spectroscopy (EDAX) were performed. Chemical analysis for mercury was conducted on 12 of the animals by atomic absorption spectrophotometry. Nine animals were found to have excessive lipofuscin in both liver and kidney. Four of these nine animals also exhibited active liver disease (fat globules, central necrosis, lymphocytic infiltrates) whereas, of the animals without the excessive pigment, only one animal had an active liver lesion. EM and EDAX showed electron-dense amorphous material presumably within lysosomes to be Hg with no deposits on mitochondrial or nuclear membranes noted. Age relationship to portal pigment deposition was positive. Liver mercury concentrations ranged from 0.01 to 443 μg/g of wet weight with all animals having liver pigment yielding values of or above 61 μg/g, whereas all animals lacking pigment had values of or below 50 μg/g. The evidence suggests that the excessive pigment accumulation is related to toxic effects of Hg and presents as increased active liver disease.

INTRODUCTION

In the course of routine postmortem studies of Atlantic Bottlenose Dolphins (*Tursiops truncatus*) stranded on the southwest coast of Florida, certain unusual histopathologic features of the liver and kidney were noted in many animals. These consisted of large deposits of a brown pigment in the portal areas of the liver, with a similar pigment present conspicuously in the liver cells and in the cells of the proximal convoluted tubules of the kidney. As these two organs are involved in mercury (Hg) biotransfer and excretion (Clarkson, 1972) and as Hg is especially concentrated in the liver (Honda *et al.*, 1983; Thompson, 1990), investigations examining a possible implication of this substance in the pathogenesis of the liver abnormalities are reported here.

MATERIALS AND METHODS

The Mote Marine Laboratory is notified by the Florida Marine Patrol of all cetacean strandings between Tampa Bay and Charlotte Harbor on the west coast of Florida. The animals range from living creatures to dead animals in advanced decomposition. Tissues whose preservation was adequate for histologic study were fixed in 10% buffered formalin solution, embedded in paraffin, sectioned at 6 μm, and stained with hematoxylin–eosin for general survey. All cases were examined for ferric iron using the

0147-6513/93 $5.00

Gomori iron reaction (Luna, 1968). The Schmorl reaction for lipofuscin was performed using the technique of Pearse (1960).

Determination of age. The age of each animal was determined by preparing thick, undecalcified sections of teeth. Growth lines were visualized by indirect lighting, photographed, and counted (Hohn *et al.,* 1989).

Electron microscopy. Formalin-fixed samples of liver were cut into 1-cm^3 cubes, and postfixed for 1 hr in 1.5% glutaraldehyde in 0.1 *M* Na cacodylate buffer (pH 7.4). Then the tissues were fixed in 1% OsO_4 in 0.1 *M* Na cacodylate buffer for 1 hr, dehydrated in ascending grades of ethanol, and embedded in Poly-Bed 812 epoxy resin. Ultrathin sections (180 nm thick) were cut with diamond knives on a LKB Nova ultramicrotome, collected on uncoated 200 mesh copper grids, and coated with carbon before examination and X-ray analysis.

X-ray spectroscopy (EDAX) was performed with scanning mode, and spectra were collected with a Kevex 8000 energy-dispersive X-ray spectrometer installed on a Hitachi H-600 electron microscope. The probe current was allowed to reach a stable level (usually 1.0 to 1.5 nA), and then the spectra were collected with a probe of 10 nm at 100 kV for 100 sec. Peak identification and semiquantification were performed using a thin film analysis based on the standardless method (Cliff and Lorimer, 1975).

Determination of mercury concentration in liver. Analysis for Hg was done by an acid-permanganate digestion followed by cold-vapor atomic absorption using a Thermo-Jarrell-Ashe atomic absorption spectrophotometer.

RESULTS

In a series of 18 dolphins with adequate liver preservation studied between January 1987 and May 1991, it was noted that 9 animals had heavy deposits of a coarsely granular pigment within the connective tissue of the portal areas of the liver (Fig. 1). These same animals also had similar deposits within hepatocytes. The granules, when discrete, were a golden brown and when aggregated were a dark brown. Intracellular deposits tended to be less aggregated, whereas in the portal tracts aggregated granules predominated. Occasional cells appeared to be so crowded with granules that the nucleus could no longer be seen; aggregated granules predominated in these cells. In these animals the cells of the proximal tubules of the kidney also contained smaller numbers of the discrete golden brown granules, but not the aggregates. No significant amount of pigment was found in either liver or kidney of the remaining 9 animals.

The pigment, both aggregated and unaggregated, had the general appearance of lipofuscin. It did not contain ferric iron, thus excluding the possibility that it was hemosiderin. The pigment displayed a strong Schmorl reaction, however. The latter consists of the reduction of ferric ferricyanide to ferric ferrocyanide by components of some pigments: melanin and argentaffin granules (which have a very different appearance from those described above) and lipofuscin and tissue components containing sulfhydryl groups (Pearse, 1960).

In four of the nine pigment-containing livers, active liver disease, in addition to pigment deposit, was present. Two animals (MML9004 and MML9108) had extensive fat globules within hepatocytes in all lobular locations. One animal (MML9001) had widespread central necrosis with fat globules in adjacent hepatocytes. A fourth animal (MML9011) had abundant lymphocytic infiltrates among hepatocytes adjacent to the portal tracts. In three other animals (MML8912, MML8917, MML8918) no definitive

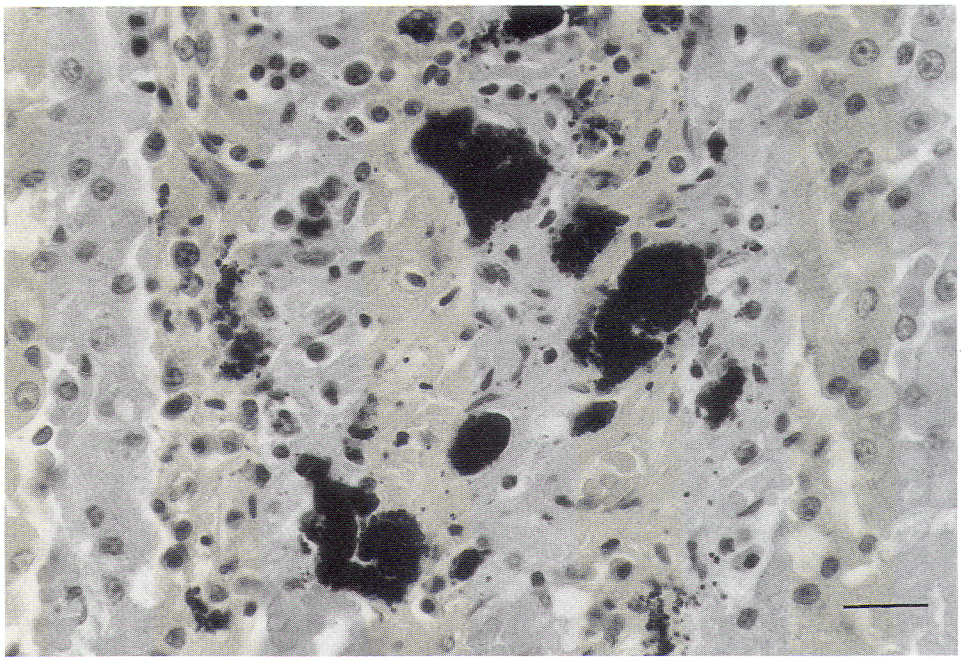


FIG. 1. Animal MML8918. Portal area of liver showing abundant deposit of dark granular pigment (H&E). Bar = 70 μm.

lethal process could be established. In the group without liver pigment, only one animal (MML8903) showed an active liver lesion. This consisted of fat globules limited to the centrilobular hepatocytes. It is of interest that this animal had the highest Hg level in the nonpigmented group. A definitive nonhepatic cause of death was established in six of the remaining eight animals (Table 1).

Examination of a specimen of liver containing pigment by electron microscopy and EDAX revealed electron-dense amorphous material presumably within lysosomes (Fig. 2a). This material corresponded to the granular intracellular deposits noted by light microscopy. EDAX revealed this material to be Hg (Fig. 2b). No other heavy metal was seen except for smaller amounts of iron, presumably in the ferrous state. Hg appeared to be present only in the lysosomes; no deposits on mitochondrial or nuclear membranes were noted. The extracellular deposits within the connective tissue of the portal tracts likewise were rich in Hg.

A comparison was made between the ages of the animals having extensive portal pigment deposits and those lacking such deposits. This revealed that these deposits were found more frequently in older animals (Table 1). Animals with pigment ranged from 6 to 21 years, whereas those without pigment ranged from 0.5 to 15 years.

In 12 of the animals, liver tissue was available for the determination of the concentration of Hg. Concentrations of Hg ranged from less than 0.01 to 443 μg/g of wet weight (Table 1). All animals having liver pigment had values of or above 61 μg/g, whereas all animals lacking pigment had values of or below 50 μg/g. The means were 245 and 24, respectively. For the difference in means, $P < 0.01$ (99% confidence level).

TABLE 1

COMPARISON OF ANIMALS WITH AND WITHOUT PIGMENT IN THE PORTAL TRACTS

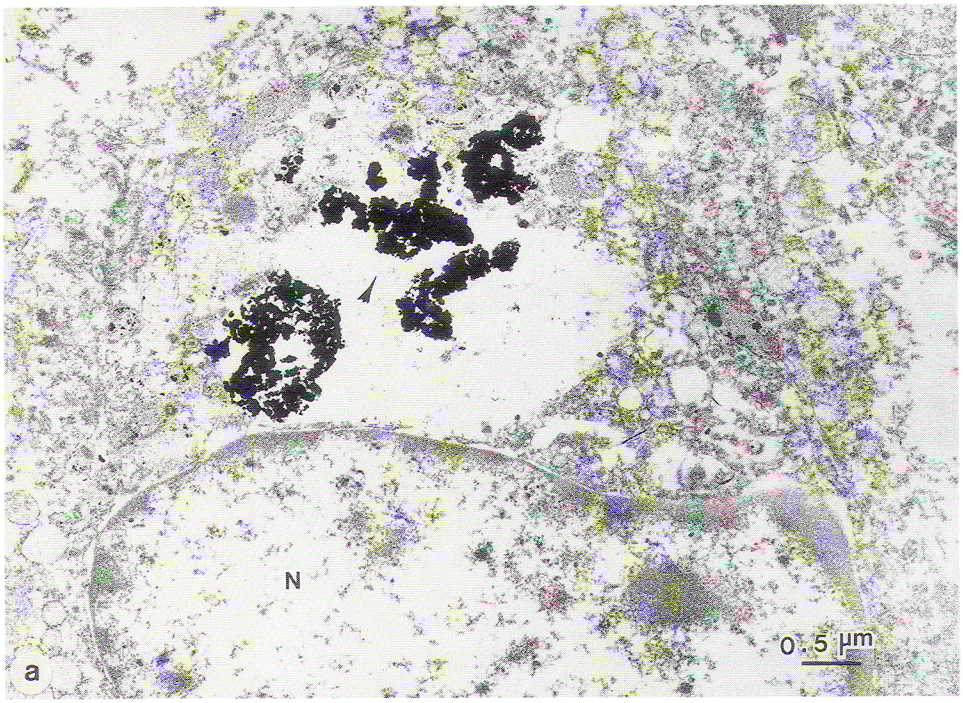
MML	Age (years)	Hg (μg/g)	Remarks
		Pigment	
9014	21	443	Lymphangiomyoma
9108	15	178	Fatty liver
9001	15	266	Central necrosis
8804	14	355	Aspiration
9011	14	NA	Hepatitis
8912	12	NA	NDLP
8918	11	166	NDLP
8917	10	NA	NDLP
9004	6	61	Fatty liver
		No pigment	
8902	15	38	Intestinal obstr.
8903	8	50	Fatty liver
8701	6	NA	Lung trauma
8702	6	NA	Lung abscess
8826	5	17	Abscesses
8715	3	35	NDLP
9006	1	7	Suffocation
8809	0.5	NA	NDLP
8901	0.5	<0.01	Pneumonia

Note. NA, not available; NDLP, definite lethal process not identified.

DISCUSSION

The appearance of the granules, both within cells and in the portal tracts, together with the absence of ferric iron, and a positive reaction with Schmorl reagents strongly suggest that the pigment is lipofuscin. Lipofuscin is believed to be derived from damaged subcellular membranes and forms an indigestible residue within lysosomes (Robbins *et al.,* 1981). It occurs normally in certain organs with aging, but is confined within cells. The finding of large extracellular deposits in the portal connective tissue suggests an enhanced accumulation of this material within the hepatocytes, implying either an excessive degree of breakdown of cell organelles or an impaired digestion of the breakdown products, or both. Although the predominance of older animals in the group with liver pigment suggests an age relationship, evidence against a simple correlation of pigment accumulation with age is provided by two animals (MML8902 and MML8903) which showed no pigment, yet were well within the age range of those that did have pigment accumulation. Both these animals had relatively low liver Hg values. The tendency of older animals to have higher levels of Hg in the liver, suggesting a chronic cumulative process, is in agreement with similar correlations for *Stenella coeruleoalba* (Honda *et al.,* 1983) and for *Phocoena phocoena* (Gaskin *et al.,* 1979).

Several lines of evidence suggest that this excessive pigment accumulation is related to toxic effects of Hg. First, pigment in the liver correlates strongly with Hg concen-



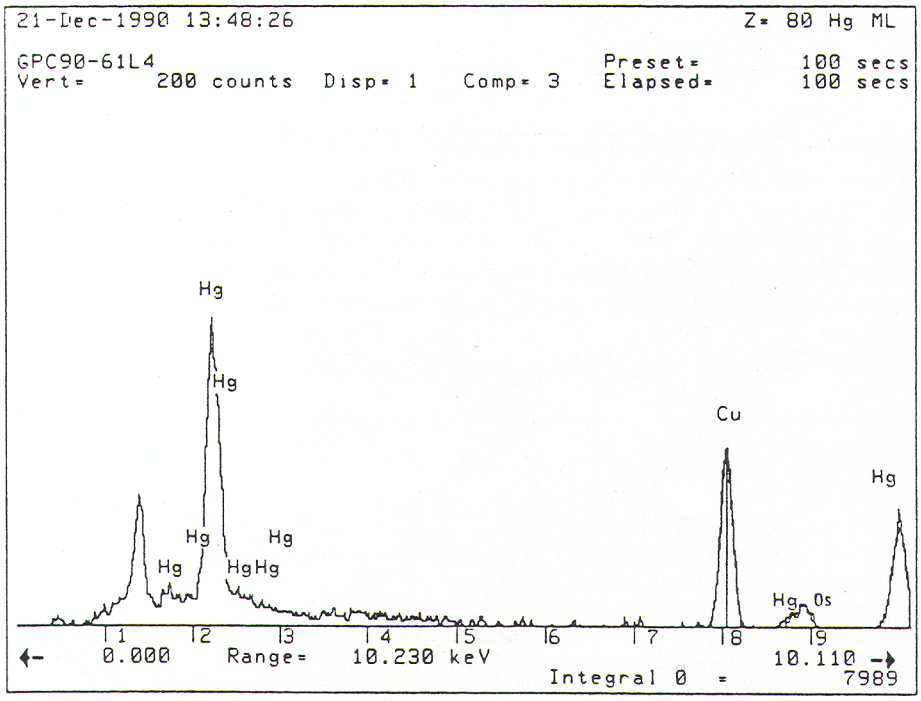


FIG. 2. (a) Animal MML8918. Representative electron micrograph of a hepatocyte showing dense osmiophilic deposits (arrow) near the nucleus (N). The deposits are presumably within lysosomes, but this cannot be clearly seen because of the poor preservation of the cellular organelles. (b) Energy dispersive X-ray spectroscopy of the osmiophilic cytoplasmic deposits shown in a. Note the high peak for Hg. The Cu peak is due to the copper grid, and the Os peak to the fixative.

tration to the extent that there is no overlap in the Hg values for the pigmented and nonpigmented groups. The correlation of pigment with age is less clear-cut. Second, Hg, and no other toxic heavy metal, was found to regularly accompany pigment both in the lysosomes and free in the portal tracts. The tendency of Hg to accumulate in lysosomes of liver cells has been noted previously (Baatrup and Danscher, 1987). Third, Hg has a high affinity for sulfhydryl groups (Hughes, 1957; Choi, 1984) and is almost entirely bound to protein in the body (Clarkson, 1972). Hg appears to bind to active membrane sites, following which it is rapidly internalized (Foulkes, 1988). Thus it is not surprising that Hg associated with damaged organelle membranes might be found within lysosomes. It has been demonstrated that Hg also inhibits the activity of lysosomal digestive enzymes (Madsen and Christensen, 1978). This would suggest that reduced degradation of proteins also might lead to excessive accumulation of lipofuscin within cells. The excessive accumulation of this indigestible residue probably results in death of the containing cell, leaving extracellular pigment deposited in the portal tract. How the pigment arrives in the portal tract is not entirely clear. Possible mechanisms are transport via macrophages or, alternatively, extension of portal connective tissue to include dying cells packed with pigment.

If pigment accumulation is indeed evidence of Hg effect, is it significant with regard to the health of the animal? The fourfold increase in active liver disease in the group with liver pigment suggests that it may be significant. In particular, the presence of fatty liver in the majority of cases probably reflects injury to the membranes of the endoplasmic reticulum, thus interfering with fat metabolism. Fatty change may forbode cell death (Robbins *et al.*, 1981) and indeed was accompanied by cell death in one instance (MML9001).

CONCLUSION

The concentration of Hg found in the livers of our small series of animals generally correlates with that found in *S. coeruleoalba* off the east coast of Japan (Honda *et al.*, 1983). These values ranged from 1.7 to 485.0 μg/g of wet weight. In all cetaceans reviewed (Thompson, 1990), the concentration of Hg in the liver was several times that in the kidney. Although concentrations of Hg in the kidney were not quantitated in the present series, lipofuscin was far more abundant in the liver than in the kidney.

ACKNOWLEDGMENTS

The authors are grateful for the generous assistance of Sarasota Pathology and Mrs. Irene Stein with the histological preparations for light microscopy.

REFERENCES

BAATRUP, E., AND DANSCHER, G. (1987). Cytochemical demonstration of mercury deposits in trout liver and kidney following methyl mercury intoxication: Differentiation of two mercury pools by selenium. *Ecotoxicol. Environ. Saf.* **14,** 129–141.

CHOI, B. H. (1984). Cellular and subcellular demonstration of mercury in situ by modified sulfide-silver technique and photoemulsion histochemistry. *Exp. Mol. Pathol.* **40,** 109–121.

CLARKSON, T. W. (1972). The pharmacology of mercury compounds. *Annu. Rev. Pharmacol.* **12,** 375–406.

CLIFF, G., AND LORIMER, G. W. (1975). The quantitative analysis of thin specimens. *J. Microsc.* **103,** 203–207.

FOULKES, E. C. (1988). On the mechanism of transfer of heavy metals across cell membranes. *Toxicology* **52,** 263–272.

GASKIN, D. E., STONEFIELD, K. I., SUDA, P., AND FRANK, R. (1979). Changes in mercury levels in harbour porpoises from the Bay of Fundy, Canada and adjacent waters during 1969–1977. *Arch. Environ. Contam. Toxicol.* **8,** 733–743.

GIBLIN, F. J., AND MASSARO, E. J. (1973). Pharmacodynamics of methyl mercury in the rainbow trout (*Salmo gairdneri*): Tissue uptake, distribution and excretion. *Toxicol. Appl. Pharmacol.* **24,** 81–91.

HOHN, A. A., SCOTT, M. D., WELLS, R. S., SWEENEY, J. C., AND IRVINE, A. B. (1989). Growth layers in teeth from known-age free-ranging bottlenose dolphins. *Mar. Mammal Sci.* **5,** 315–342.

HONDA, K., TATSUKAWA, R., KAZUOMI, I., MIYAZAKI, N., AND FUJIYAMA, T. (1983). Heavy metal concentrations in muscle, liver and kidney tissue of striped dolphin, *Stenella coeruleoalba,* and their variations with body length, weight, age and sex. *Agric. Biol. Chem.* **47,** 1219–1228.

HUGHES, W. L. (1957). A physicochemical rationale for the biologic activity of mercury and its compounds. *Ann. N.Y. Acad. Sci.* **65,** 454–460.

LOCKHART, W. L., UTHE, J. F., KENNEY, A. R., AND MEHRLE, P. M. (1972). Methyl mercury in northern pike (*Esox lucius*): Distribution, elimination, and some biochemical characteristics of contaminated fish. *J. Fish Res. Board Can.* **29,** 1519–1523.

LUNA, L. G. (Ed.) (1968). *Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology.* 3rd ed. Mcgraw–Hill, New York.

MADSEN, H. M., AND CHRISTENSEN, E. I. (1978). Effects of mercury on lysosomal protein digestion in the kidney proximal tubule. *Lab. Invest.* **38,** 165–174.

PEARSE, A. G. E. (1960). *Histochemistry: Theoretical and Applied.* Little, Brown, Boston.

ROBBINS, S. L., ANGELL, M., AND KUMAR, V. (1981). *Basic Pathology,* 3rd ed., pp. 17–18, 24. Saunders, Philadelphia.

THOMPSON, D. R. (1990). Metal levels in marine vertebrates. In *Heavy Metals in the Marine Environment* (R. W. Furness, and P. S. Rainbow, Eds.), pp. 164–166. CRC Press, Boca Raton, FL.